



TITLE:

PDK1 Is a Regulator of Epidermal Differentiation that Activates and Organizes Asymmetric Cell Division

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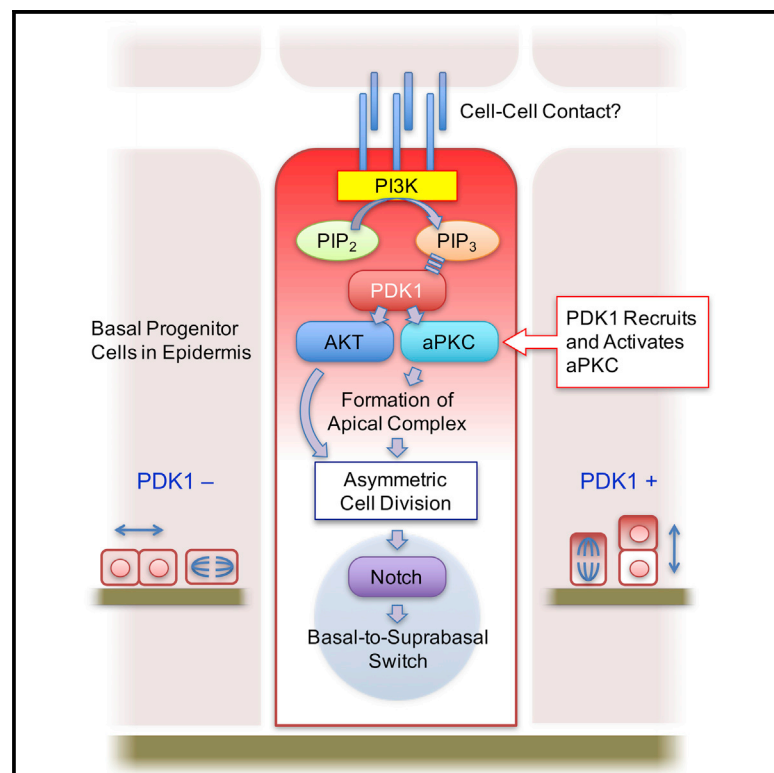
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PDK1 Is a Regulator of Epidermal Differentiation that Activates and Organizes Asymmetric Cell Division

Graphical Abstract



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In Brief

Dainichi et al. demonstrate that PDK1 plays a critical role in asymmetric cell division, wherein PDK1 regulates the activation of AKT, as well as the activation and redistribution of aPKC. PDK1 signaling from the apical side of basal keratinocytes regulates the basal-to-suprabasal switch in epithelial stratification and the Notch-dependent differentiation program.

Highlights

- Loss of PDK1 impairs epidermal ACD, differentiation, and stratification
- PIP₃-PDK1 signaling occurs preferentially at the apical side of dividing basal cells
- PDK1 is crucial for activation and recruitment of the apical complex in basal cells
- Restoration of Notch rescues the defective differentiation of keratinocytes in vitro



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Report

PDK1 Is a Regulator of Epidermal Differentiation that Activates and Organizes Asymmetric Cell Division

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SUMMARY

Asymmetric cell division (ACD) in a perpendicular orientation promotes cell differentiation and organizes the stratified epithelium. However, the upstream cues regulating ACD have not been identified. Here, we report that phosphoinositide-dependent kinase 1 (PDK1) plays a critical role in establishing ACD in the epithelium. Production of phosphatidylinositol triphosphate (PIP₃) is localized to the apical side of basal cells. Asymmetric recruitment of atypical protein kinase C (aPKC) and partitioning defective (PAR) 3 is impaired in PDK1 conditional knockout (CKO) epidermis. PDK1^{CKO} keratinocytes do not undergo calcium-induced activation of aPKC or IGF1-induced activation of AKT and fail to differentiate. PDK1^{CKO} epidermis shows decreased expression of Notch, a downstream effector of ACD, and restoration of Notch rescues defective expression of differentiation-induced Notch targets *in vitro*. We therefore propose that PDK1 signaling regulates the basal-to-suprabasal switch in developing epidermis by acting as both an activator and organizer of ACD and the Notch-dependent differentiation program.

INTRODUCTION

Generation of three-dimensional tissues with different cell types characterizes the development of all organs. This process is triggered by intrinsic or extrinsic cues, and it is coupled to the generation of different cells from common progenitors through a process known as asymmetric cell division (ACD) (Knoblich, 2010). ACD drives the development and differentiation of the epidermis in mammals (Ray and Lechler, 2011; Williams et al.,

2011), where a balance between symmetric and asymmetric divisions generates a tissue of the correct surface area and thickness. The differentiation of the epidermis begins with the stem cells located within the basal layer (Fuchs, 2009), and ACD in a perpendicular orientation relative to the basement membrane promotes cell differentiation mediated by several transcriptional regulators and organizes the stratified epithelium (Arnold and Watt, 2001; Hu et al., 1999; Lopez et al., 2009; Mills et al., 1999; Rangarajan et al., 2001; Takeda et al., 1999; Wang et al., 2008). However, both the molecular cues that trigger organization of the apical complex during ACD and the signaling pathways that drive activation of apical complex components remain to be defined.

Phosphoinositide-dependent kinase 1 (PDK1) is a serine/threonine kinase of the AGC kinase group. The kinase activity of PDK1 depends on phosphatidylinositol 3-kinase (PI3K), a key intermediate in signaling pathways including those from growth factor receptors and adhesion molecules. Substrates of PDK1, including AKT and the protein kinase C (PKC) isoforms, regulate a number of essential cell functions (Pearce et al., 2010). In particular, atypical PKC (aPKC) is involved in cell polarity and ACD (Knoblich, 2010). However, in mammalian epidermis, the role of aPKC remains unclear. There are two aPKC isoforms in mammals, PKC ζ and PKC λ . Loss of PKC ζ reportedly has no effect on epidermal differentiation (Leitges et al., 2001). In contrast, epidermal loss of PKC λ results in disruption of ACD, but with enhanced ACD and defective stem cell homeostasis (Niessen et al., 2013). However, in these studies, conformation of the apical complex, which is a critical cellular event at the beginning of ACD, was not affected by the absence of PKC λ as partitioning defective (PAR) 3 and other components were still recruited to the apical complex. These findings suggest either redundancy between aPKC isoforms or aPKC-independent mechanisms of apical complex assembly and ACD in epidermis.

In addition to phosphorylating PKC proteins, PDK1 also may facilitate the function of PKC proteins by acting as a scaffold molecule bridging PKC and downstream substrates. During

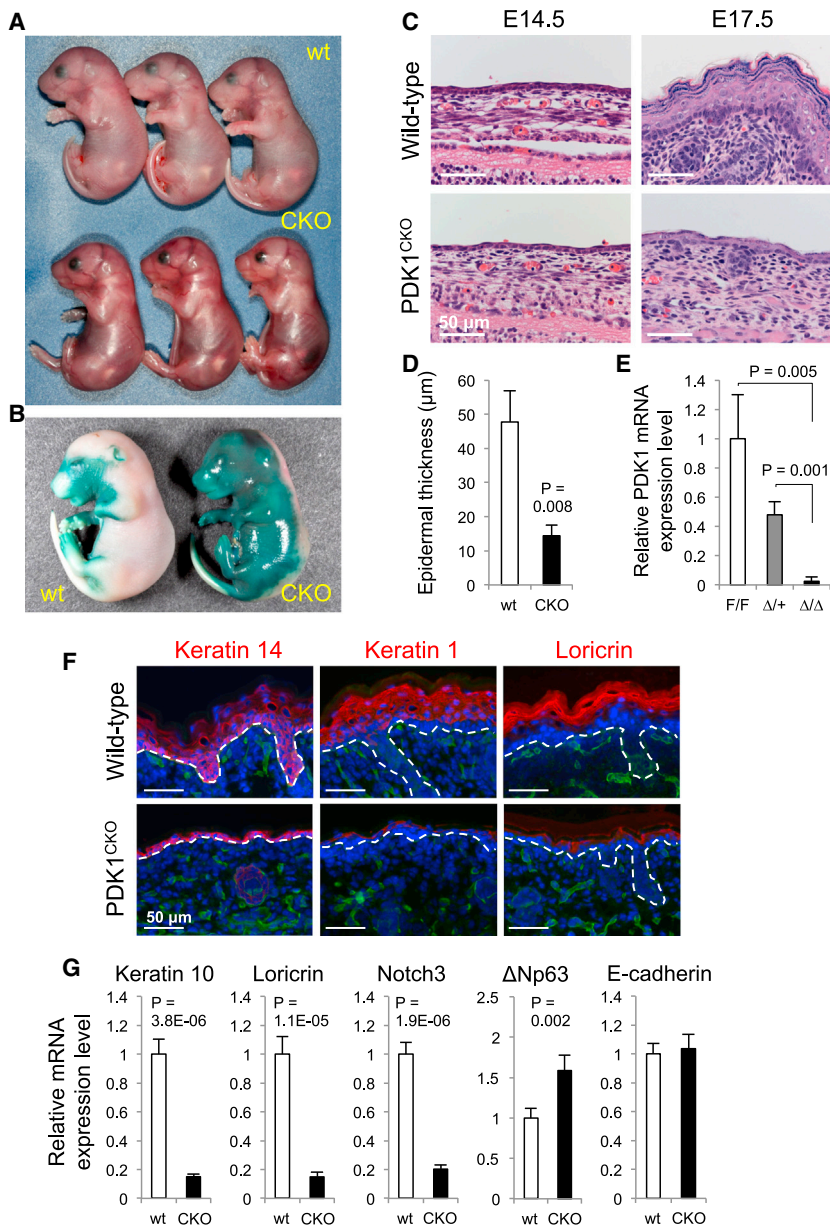


Figure 1. Phenotype of PDK1-Deficient Epidermis

(A) Gross appearance of K14-Cre^{tg/+} PDK1^{Flox/+} wild-type (WT, top) and K14-Cre^{tg/+} PDK1^{Flox/Flox} CKO E17.5 embryos (bottom) is shown.

(B) Dye exclusion assay of E17.5 embryos. Blue staining shows impaired epidermal barrier function of PDK1^{CKO} (right) compared to the WT (left) mice.

(C) Histology of dorsal skin from E14.5 and E17.5 embryos with H&E staining. WT (top) and PDK1^{CKO} (bottom) mice in which hair follicles appeared to be arrested in hair peg stage on E17.5 are shown. All scale bars, 50 μ m.

(D) Thickness of epidermis is shown for WT (white column, n = 30) and PDK1^{CKO} (black column, n = 45) E17.5 embryos (error bars, SD).

(E) PDK1 gene expression from newborn epidermis of PDK1^{Flox/Flox} (F/F, white column), K14-Cre^{tg/+} PDK1^{Flox/+} (Δ +, gray column), and K14-Cre^{tg/+} PDK1^{Flox/Flox} (Δ Δ , black column) mice was evaluated by qPCR (error bars, SD; n = 3).

(F) IF of WT (top) and PDK1^{CKO} (bottom) mouse dorsal skin on E17.5, performed with anti-keratin-14, keratin-1, or loricrin (red signals, respectively), anti-laminin antibodies (green), and DAPI staining (blue). White dashed lines indicate basement membrane. All scale bars, 50 μ m.

(G) Gene expression profiles from newborn epidermis of WT (white columns) and PDK1^{CKO} (black columns) mice were evaluated by qPCR (error bars, SD; n = 4).

triggers PI3K, leading to the asymmetric accumulation of the lipid effector phosphatidylinositol triphosphate (PIP₃). Enrichment of PIP₃ at the apical side also leads to recruitment and activation of PDK1, thus establishing an asymmetric signaling pathway in differentiating cells. Deletion of PDK1 abolishes ACD and both activation of downstream signaling-pathway components, including AKT, glycogen synthase kinase (GSK)-3 β , and aPKC, and polarization of components of the apical complex. Thus, PDK1 is essential for both activation and asymmetric organization of key regulators of ACD. Consequently, loss of PDK1 leads

to dramatically attenuated differentiation and stratification of the epidermis, with disrupted barrier function and perinatal lethality.

RESULTS AND DISCUSSION

PDK1 Has a Non-redundant Role in Epidermal Differentiation and Stratification

To test whether PDK1 has any role in keratinocyte differentiation, we generated epidermis-specific PDK1 conditional knockout (CKO) mice using K14-Cre. While PDK1 ^{Δ +/+} epidermis from K14-Cre^{tg/+} PDK1^{Flox/+} showed a wild-type phenotype, K14-Cre^{tg/+} PDK1^{Flox/Flox} (PDK1^{CKO}) mice exhibited thin and shiny epidermis (Figure 1A). They showed hypoplasia of vibrissae

T cell receptor signaling, which is a highly polarized signaling process that can trigger ACD (Chang et al., 2007), PDK1 facilitates signaling by acting as a structural platform that activates PKC θ and links PKC θ to downstream substrates (Lee et al., 2005; Park et al., 2009). Interestingly, a small molecule screening study suggested that activation of PDK1 enhances embryonic stem cell (ESC) reprogramming (Zhu et al., 2010). Therefore, although the role of PDK1 in ACD and cell differentiation had not been previously investigated, we hypothesized that PDK1 might serve as a key organizer of the apical complex during ACD. We therefore investigated the function of PDK1 through conditional deletion of PDK1 in the epidermis.

We now report that PDK1 plays a critical role in the establishment of ACD in the epidermis. We proposed that apical signaling

although they still developed rudimentary vibrissal follicles. PDK1^{CKO} mice were unable to ingest milk, although we did not detect any cleft palate or obstruction in esophagus by dye inoculation test or histology. The PDK1^{CKO} mice died within several hours of birth, which, based on gross appearance, we suspected was due to excessive transepidermal water loss. An outside-in dye exclusion assay revealed that the barrier function of PDK1-deficient epidermis was impaired (Figure 1B). Histology of skin specimens showed that the stratification of epidermis from PDK1^{CKO} was dramatically attenuated at embryonic day 17.5 (E17.5), although the development of wild-type and PDK1^{CKO} epidermis was comparable at E14.5 (Figure 1C). This reduction of thickness in PDK1^{CKO} epidermis also was evaluated quantitatively (Figure 1D). Expression of PDK1 was nearly abolished in epidermis from PDK1^{CKO} mice (Figure 1E), suggesting that the phenotype was due to the absence of PDK1 in the epidermis.

Immunofluorescence (IF) analysis revealed that the thin epidermis of PDK1^{CKO} mice expressed normal levels of a basal cell marker keratin-14, whereas expressions of a spinous cell marker, keratin-1, and a granular cell marker, loricrin, were markedly decreased (Figure 1F). The PDK1^{CKO} epidermis expressed keratin-6, which is prominent in regeneration and pathologic states (Figure S1A). However, no clear change in cellularity of the basal cell layer was detected (Figure 1F). Furthermore, we were unable to detect a significant decrease in cell proliferation or increase in apoptosis in the inter-follicular epidermis (IFE) of PDK1^{CKO} mice (Figures S1B and S1C), consistent with findings in PDK1-null embryos (Lawlor et al., 2002). Analysis of cell number during primary culture was comparable between wild-type and PDK1-deficient keratinocytes (Figure S1D); however, the size of PDK1-deficient keratinocytes was smaller than that of wild-type cells (Figure S1E), consistent with previous observations in other tissues (Hashimoto et al., 2006; Lawlor et al., 2002).

We used qPCR to analyze expression of genes implicated in epidermal differentiation. Consistent with the IF results, expressions of a spinous cell marker, keratin-10, and granular cell markers, involucrin and loricrin, were markedly suppressed in PDK1-deficient mice (Figures 1G and S1F). Expressions of Notch, an essential regulator of keratinocyte differentiation following ACD (Rangarajan et al., 2001; Williams et al., 2011), and its transcriptional target HES1, also were suppressed at the mRNA level (Figures 1G and S1F). In contrast, expression of Δ Np63, a dominant-negative isoform of p63 that represses key genes and maintains basal cells in the progenitor state (Candi et al., 2007; Fuchs, 2009), was slightly increased in PDK1^{CKO} epidermis (Figure 1G), likely attributable to a decrease in Δ Np63^{low}, differentiated cells in the PDK1^{CKO} epidermis. However, levels of other transcriptional regulators of keratinocytes, such as I κ B kinase α (IKK α) (Hu et al., 1999; Takeda et al., 1999), AP-2 (Wang et al., 2008), and C/EBP (Lopez et al., 2009), were not affected (Figure S1F). Taken together, these results suggest that PDK1 is necessary for differentiation of basal progenitor cells into suprabasal spinous cells, whereas development of basal keratinocytes and organization of the basal layer are PDK1 independent. As a result, in the absence of PDK1, epidermal development is arrested at approximately the E14.5 developmental stage.

Basal Cell Asymmetric Division Is Decreased in PDK1^{CKO} Epidermis

To further analyze the defect in PDK1^{CKO} epidermis, we evaluated cell division in wild-type and PDK1^{CKO} skin specimens with H&E staining. Consistent with a previous study (Lechler and Fuchs, 2005), normal keratinocyte differentiation and stratification from basal cells occurred in an asymmetric manner; however, in E17.5 PDK1-deficient epidermis, most cell divisions were symmetric in a horizontal orientation (Figures 2A and 2B). Surprisingly, despite the increased rate of symmetric division of basal cells in vivo and normal proliferation of PDK1^{CKO} keratinocytes in vitro, we were unable to detect significant changes in the cellularity of the basal layer. Given the defect in ACD in PDK1^{CKO} epidermis, we examined the distribution of PDK1 in dividing basal cells in wild-type mice. Although we could not detect significant polarization of PDK1 in metaphase or anaphase cells (Figure S2A), in telophase basal cells that were undergoing division in a perpendicular orientation, PDK1 was enriched in the apical daughter cells (Figure 2C). In cells dividing in a horizontal orientation, in contrast, PDK1 was symmetrically distributed to both daughter cells. Hence, our results suggest that PDK1 is required for ACD in a vertical orientation, perpendicular to the basement membrane, and that PDK1 is enriched in the resulting apical daughter cell.

PIP₃-PDK1-aPKC Signaling at the Apical Side Cues ACD

PDK1 is a key intermediate in signaling downstream of PI3K (Pearce et al., 2010). Cell-cell contact stimuli, governed by cadherin and desmoglein molecules, are one, though likely not the only, candidate for activating the PI3K pathway in keratinocytes (Calautti et al., 2005). Indeed, we found that PDK1 and E-cadherin are co-localized at sites of cell-cell contact in wild-type E17.5 dorsal epidermis (Figure S2B). E-cadherin directly activates PI3K and induces keratinocyte differentiation in a calcium-dependent manner (Calautti et al., 2005). Cell-cell contact through E-cadherin also is required for germline stem cell spindle orientation in *Drosophila* (Inaba et al., 2010). While depletion of PDK1 in epidermis did not affect the expression of the E-cadherin gene (Figure 1G), phosphorylation of AKT, a substrate of PDK1, was, as expected, absent in the dorsal epidermis of newborn mice (Figure 3A). Consistent with the distribution of E-cadherin and with previous studies (Calautti et al., 2005), IF revealed that phosphorylated AKT and also PDK1 were localized at cell-cell contact sites in newborn wild-type suprabasal cells in vivo. Phosphorylation of AKT at cell-cell contact sites also was present in vitro (Figure S2D).

Besides cell-cell contact stimuli, cell-matrix stimuli from the basement membrane through molecules such as laminin also could activate the integrin-PI3K pathway (Pearce et al., 2010) in basal progenitor cells. To identify the crucial cues for PDK1-dependent ACD in epidermis, we evaluated the distribution of PIP₃ in basal cells. We found that PIP₃ is predominantly localized to the apical side, but not the basolateral side, of basal cells in wild-type epidermis (Figure 3B). PIP₃ also predominantly was produced at the apical side of basal cells in PDK1^{CKO} E17.5 epidermis (Figure 3B), suggesting that apical activation of PI3K and the upstream cell-cell contact stimuli are maintained in the basal cells even in the absence of

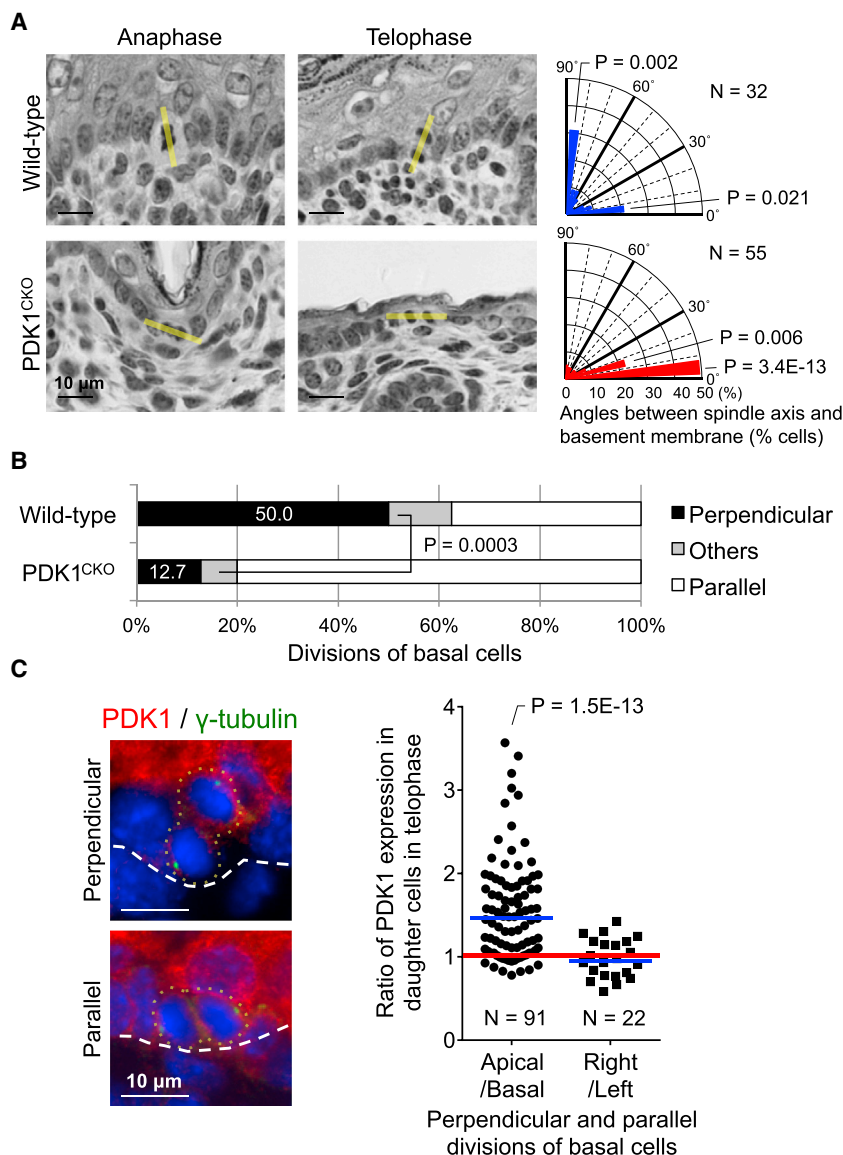


Figure 2. Defective ACD in PDK1-Deficient Epidermis

(A) Representative basal cell mitoses in dorsal epidermis from WT (top) and PDK1^{CKO} (bottom) E17.5 embryos with H&E staining. Yellow lines indicate spindle orientation in anaphase (left) and telophase (middle). The spindle orientation relative to the basement membrane was observed in multiple fields of view and is presented graphically (right). All scale bars, 10 μ m.

(B) Percentages of cell divisions in WT and PDK1^{CKO} epidermis (three each) that were asymmetric or perpendicular to basement membrane ($90^\circ \pm 30^\circ$, black bars), symmetric or parallel to basement membrane ($0^\circ \pm 30^\circ$, white bars), or other (gray bars) were plotted.

(C) IF of actively dividing cells (circled with yellow dotted lines) in dorsal epidermis from three WT E17.5 embryos with anti-PDK1 (red signals) and γ -tubulin antibodies (green) with DAPI staining (blue). γ -tubulin signals indicate centrosomes in telophase. White dashed lines indicate basement membrane. Distribution of PDK1 signals in 113 pairs of WT dividing cells in telophase (91 in perpendicular, 22 in parallel) was quantified and graphed (right; blue bars, medians). All scale bars, 10 μ m.

of E14.5 basal cells was impaired in PDK1^{CKO} epidermis (Figures 3C and S2E). These results suggest that the epidermal differentiation and stratification in the PDK1^{CKO} were defective at E14.5 and earlier (Figure 1C). Notably, PDK1 exhibited a clear apical distribution in the wild-type, interphase basal cells (Figure 3C), suggesting that asymmetric PDK1 localization is not a result of, but precedes, asymmetric spindle orientation.

The distribution of aPKC is consistent with the idea that aPKC forms an apical complex early during ACD, though it is unclear whether aPKC kinase activity is required for PAR3 to enter the apical complex (Kno-

blich, 2010) or aPKC recruitment is mediated by PAR3 (Rodriguez-Boulant and Macara, 2014). In either case, PDK1 is required for the recruitment of both components of the apical complex. These results suggest that the molecular cues for ACD in basal progenitor cells are provided from the apical side through localized PI3K activation and PDK1 recruitment. Consequently, both downstream signaling pathways and the assembly of the apical complex are abolished in PDK1^{CKO} epidermis.

Lack of Calcium-Induced aPKC Phosphorylation and Differentiation in PDK1^{CKO} Keratinocytes

To further characterize the defect in PDK1-deficient keratinocytes, we evaluated the response of PDK1^{CKO} keratinocytes to calcium in vitro. Switching experimentally from low to high calcium induces cell-cell contact mediated by cadherin molecules and provides a cue for cell differentiation in keratinocytes

PDK1. An apical distribution of PIP₃ also was detected in E14.5 basal cells (Figure S2E).

We previously had found that PDK1 was essential for polarized recruitment of PKC θ to macromolecular signaling complexes during T cell activation (Lee et al., 2005). Although loss of PKC λ in keratinocytes does regulate spindle orientation, intrafollicular basal cells lacking PKC λ display increased ACD (Niessen et al., 2013). However, it is not clear if this finding is a result of compensation by PKC ζ (Vorhagen and Niessen, 2014), as PKC ζ is upregulated in keratinocytes lacking PKC λ (Niessen et al., 2013). Consistent with some method of compensation, no changes were observed in PAR3 localization in keratinocytes lacking PKC λ (Niessen et al., 2013). Therefore, we investigated the distribution of aPKC and other known components of the apical complex. Indeed, despite normal PIP₃ distribution, localization of the PDK1 substrates aPKC and PAR3 to the apical side

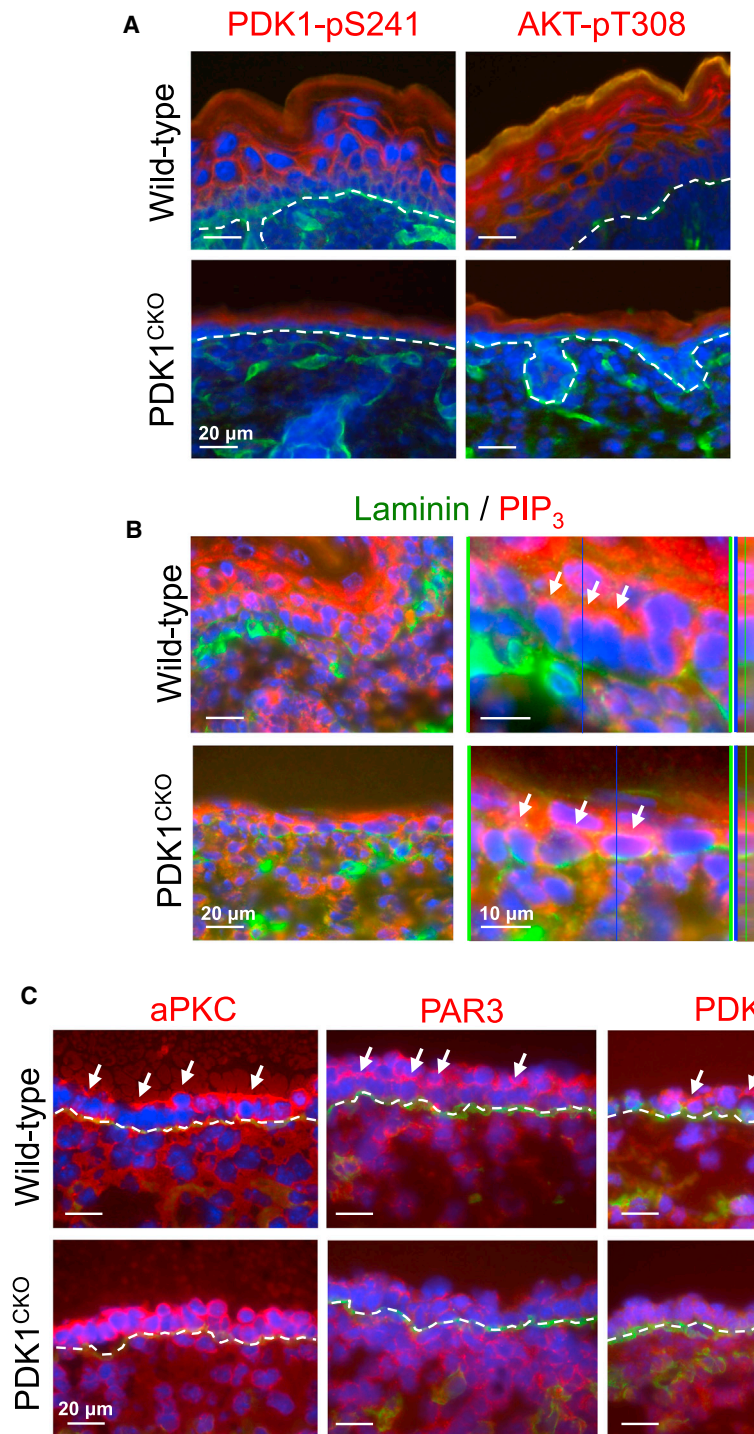


Figure 3. Absence of Molecular Cues for ACD in PDK1^{CKO} Epidermis

(A) IF of WT (left) and PDK1^{CKO} (right) newborn dorsal epidermis. Anti-phospho-PDK1 (top) and phospho-AKT (bottom) demonstrate red signals at cell-cell contact sites in the WT epidermis compared to those of isotype control (Figure S2C). Laminin, green; DAPI staining, blue. Scale bars, 20 μ m.

(B) IF of basal keratinocytes from WT (left) and PDK1^{CKO} (right) E17.5 dorsal epidermis with anti-PIP₃ (red signals at apical side of basal cells, indicated by arrows) and laminin (green) antibodies with DAPI staining (blue). The position of the XY images relative to the Z axis (green line) and the YZ images relative to the X axis (blue line) are indicated. Note that PIP₃ signals are not overlapped with laminin signals at basal side in high-power views (right panels). Scale bars, 20 μ m (left panels) and 10 μ m (right).

(C) IF of WT dorsal epidermis from E14.5 embryo (top) demonstrates apical distribution of aPKC, PAR3, and PDK1 in basal cells (red signals indicated by arrows), while they are impaired in PDK1^{CKO} epidermis (bottom). White dashed lines indicate basement membrane. Laminin, green; DAPI staining, blue. Scale bars, 20 μ m.

induced keratinocyte differentiation is not fully RBP-J κ -HES1 dependent (Rangarajan et al., 2001). At the protein level, keratin-1 and loricrin expression also was not induced in PDK1-deficient keratinocytes (Figure 4B).

MicroRNA (miRNA)-203 regulates keratinocyte differentiation by inhibiting Δ Np63 expression (Lena et al., 2008; Yi et al., 2008), which maintains stemness through its transcriptional target genes (Candi et al., 2007; Fuchs, 2009). MiRNA-203 expression in wild-type keratinocytes is induced by high calcium concentration, but, remarkably, there was no induction of miRNA-203 expression in PDK1-deficient keratinocytes (Figure 4A). However, downregulation of Δ Np63 by calcium was unaffected at both the transcriptional and protein levels (Figures 4A, 4B, and S3B), and miRNA-203 transfection did not rescue the PDK1 deficiency (Figure S3C). Expressions of Myc (Arnold and Watt, 2001) and its target

(Calautti et al., 2005; Hennings et al., 1980). The qPCR showed that keratin-10 and loricrin gene expression was not induced by high calcium in PDK1-deficient keratinocytes (Figure 4A). Induction of Notch expression was also significantly impaired in PDK1-deficient keratinocytes (Figure 4A). Induction of HES1 by calcium stimulation was not detected in wild-type or PDK1 keratinocytes in vitro (Figure S3A), suggesting that Notch-

Misu were attenuated in PDK1^{CKO}, but their levels were comparable after high-calcium culture (Figures 4A and S3A), suggesting that the decreased expression of these regulatory molecules is not responsible for the phenotype in PDK1^{CKO} keratinocytes.

As expected, PI3K phosphorylation was normal in PDK1^{CKO} keratinocytes (Figure 4C). Although aPKC is a calcium-independent kinase, PIP₃ stimulates aPKC kinase activity in the presence

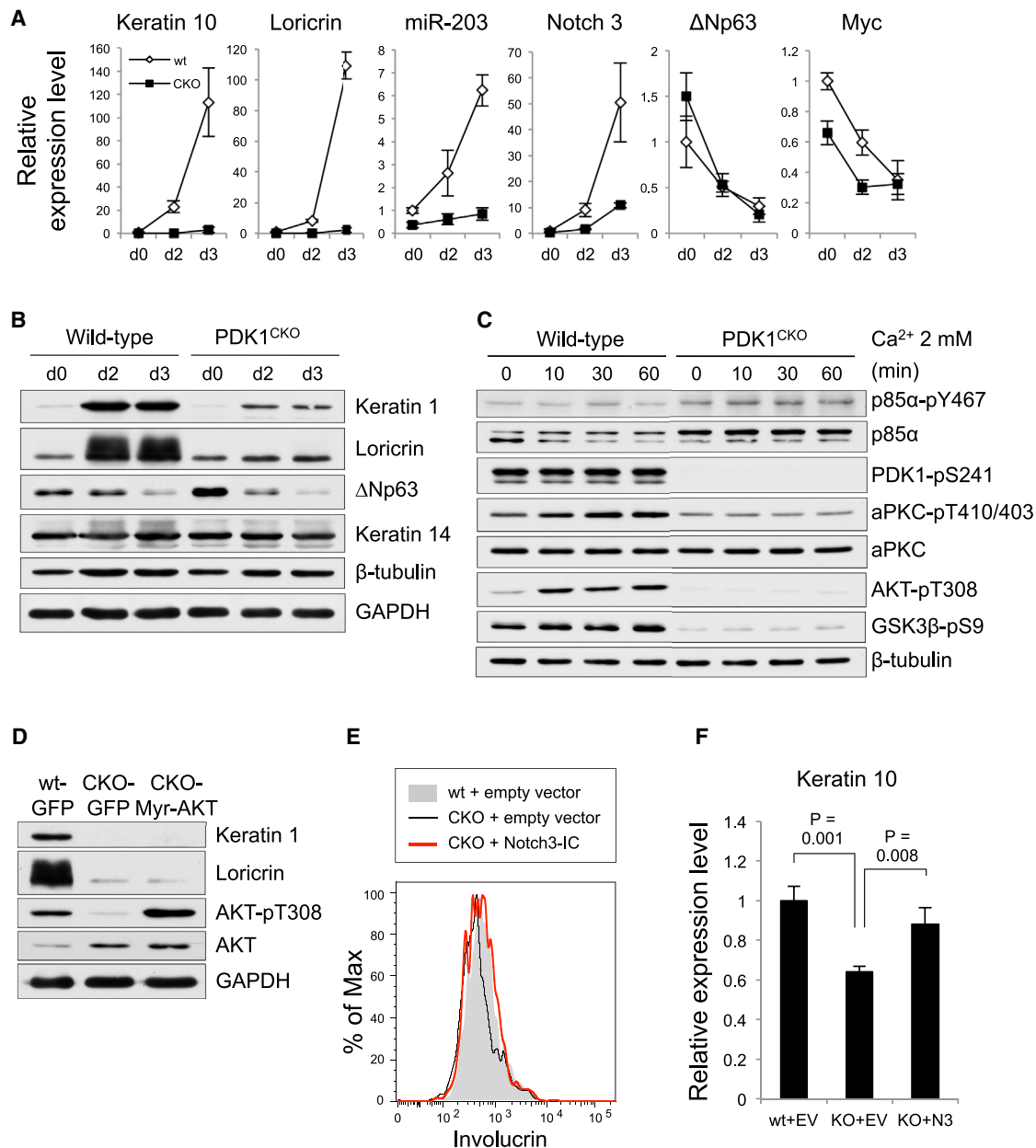


Figure 4. PDK1-Dependent Signaling in Primary Cultured Mouse Keratinocytes

(A) Keratinocytes from WT and PDK1^{CKO} mice (CKO) grown in 0.05 mM Ca²⁺ were then cultured with 1.3 mM Ca²⁺. Gene expression was evaluated by qPCR at the indicated days (error bars, SD; n = 3).

(B) Protein expression from WT and PDK1^{CKO} keratinocytes was evaluated by western blotting at the indicated days of culture with 1.3 mM Ca²⁺.

(C) WT and PDK1^{CKO} keratinocytes cultured with 0.05 mM Ca²⁺ were stimulated with 2 mM Ca²⁺ after 24-hr starvation of serum. Cells were lysed at the indicated timings and the protein expression and their phosphorylation levels were evaluated by western blotting (additional results are presented in Figure S3D).

(D) WT keratinocytes transfected with GFP adenovirus and CKO keratinocytes transfected with GFP or Myr-AKT adenovirus were cultured with 1.3 mM Ca²⁺ for 2 days. Protein expression and phosphorylation levels were evaluated by western blotting.

(E) Flow cytometry for involucrin expression. GFP⁺ WT and CKO keratinocytes were cultured with 1.3 mM Ca²⁺ for 3 days after transfection with IRES-GFP empty vector or that with Notch3 intracellular domain (ICD).

(F) Keratin-10 gene expression. GFP⁺ WT and CKO keratinocytes were cultured with 1.3 mM Ca²⁺ for 3 days after transfection with IRES-GFP empty vector (EV) or that with Notch3 ICD (N3; error bars, SD; n = 3).

of calcium (Nakanishi et al., 1993), most likely through PDK1. Consistent with this model, we found that calcium rapidly induces phosphorylation of aPKC in wild-type keratinocytes,

although it is not as rapid as AKT phosphorylation (Figure 4C). In PDK1-deficient keratinocytes, phosphorylation of AKT, GSK3 β , and aPKC was reduced or completely abolished

(Figure 4C). Insulin and insulin-like growth factor (IGF)-1 signaling has been implicated in ACD, and mice lacking insulin and IGF-1 receptors exhibit a phenotype similar to the PDK1^{CKO} mice (Günschmann et al., 2013). Consistent with the known role of PDK1, IGF-1 and epidermal growth factor (EGF)-induced phosphorylation of AKT and GSK3 β also was completely abolished in PDK1-deficient keratinocytes (Figures S3E and S3F).

A small molecule AKT inhibitor, AKTi-1/2, inhibited calcium-induced differentiation of keratinocytes in vitro similar to the reduction observed using a PDK1 inhibitor (Figures S4A and S4B). These results suggest that AKT could be the key target of PDK1 in keratinocyte differentiation. We, therefore, tested whether we could rescue the defect in PDK1^{CKO} keratinocyte differentiation through activation of AKT. We transfected PDK1^{CKO} keratinocytes with constitutively active AKT (Myr-AKT), which resulted in robust activation of AKT (Figure 4D). However, AKT activation did not rescue calcium-induced differentiation of PDK1^{CKO} keratinocytes (Figure 4D), suggesting that the AKT pathway is not sufficient to promote differentiation in the absence of PDK1. These results are consistent with observations made in AKT1/2 KO mice (Peng et al., 2003), which exhibit a stratified though thin epidermis with expression of filaggrin and keratin-10. In fact, AKT has been shown to have an inhibitory role in keratinocyte differentiation by inducing Δ Np63 in response to EGF (Barbieri et al., 2003). These results suggest that impaired differentiation of PDK1-deficient keratinocytes is not solely due to effects on AKT, but rather likely due to combinatorial defects in the activation of AKT as well as aPKC.

Activation of Notch Rescues Differentiation Defect of PDK1^{CKO} Keratinocytes

Unfortunately, we were unable to create a constitutively active form of aPKC that would allow us to overcome both impaired phosphorylation and impaired localization in the absence of PDK1. Two molecules have been suggested to be specific substrates of aPKC: lethal giant larvae (LGL) and Numb (Knoblich, 2010). The asymmetric division null mutant, Leu-Gly-Asn repeat-enriched protein (LGN) knockdown, shows impaired differentiation and stratification of epidermis, and restoration of impaired Notch signaling in the knockdown cells partially rescues the defective phenotypes, suggesting that Notch is a downstream effector of ACD (Williams et al., 2011). Inhibition of PDK1 also fully suppressed the calcium-induced expression of Notch3 mRNA in primary cultured keratinocytes in a dose-dependent manner (Figures S4C and S4D). However, expression of representative Notch response genes, such as Hes1, Hes5, Hey1, and Hey2, was not highly induced by calcium. Nevertheless, inhibition of PDK1 did result in statistically significant repression of expression of Hes5 and Hey2 (Figure S4E). These results were consistent with the analysis of PDK1^{CKO} in vivo (Figure S1F) and in vitro (Figure S3A), suggesting change in Notch-dependent transcriptional programs in PDK1^{CKO} keratinocytes.

To test whether Notch activation can rescue the impaired differentiation of PDK1^{CKO} keratinocytes, we transfected Notch3 intracellular domain (ICD) and evaluated expression of involucrin, a transcriptional target of Notch (Rangarajan et al., 2001). Impaired involucrin expression during calcium-induced differentiation in PDK1^{CKO} keratinocytes was fully restored upon Notch3

activation at the protein level (Figure 4E). Although keratin-1 protein levels were not as clearly rescued by this procedure (Figure S4F), we were able to observe that activation of Notch restored calcium-induced upregulation of keratin-10 (Figure 4F). These results suggest that, in addition to being required for ACD, PDK1 is needed for Notch-dependent transcriptional programs essential for keratinocyte differentiation.

Conclusions

We propose that PDK1 plays a critical role in epithelial differentiation and stratification. Our results highlight an essential role for PDK1 in ACD, wherein PDK1 governs both the activation and localization of key signaling pathways. PDK1 regulates the activation of AKT and the activation and redistribution of aPKC and PAR3 at the apical pole of asymmetrically dividing cells. As a result, PDK1 appears to be crucial for the early signaling that is triggered at the apical side of progenitor cells leading to cell division in a perpendicular plane to the basal membrane and ultimately to the basal-to-suprabasal switch required for development of stratified epithelium.

Our study provides additional insight into the long-standing question of whether apical or basal stimuli provide the cue for ACD in stratified epithelia. We found that PDK1 is enriched at the apical side of dividing basal cells and that this localization correlates with the enrichment of PIP₃. Therefore, although PI3K can be activated at both the lateral and basal sides of epithelial cells (McCaffrey and Macara, 2012), we propose that the activation and recruitment of PDK1 at the apical side is a PI3K-dependent process that is crucial for establishing ACD. Despite normal PIP₃ localization in cells lacking PDK1, we found that aPKC and PAR3 no longer were redistributed to the apical side of basal progenitor cells in the absence of PDK1. This suggests that establishing the apical complex is dependent on PI3K activation and recruitment of PDK1 to enriched PIP₃ at the apical side. Thus, PDK1 acts both as a scaffold, by recruiting the apical complex to the PIP₃-enriched membrane, and as a key signaling intermediate necessary for the activation of aPKC, as well as AKT. These findings are consistent with the role of PDK1 in the organization of signaling from the T cell receptor-signaling complex (Lee et al., 2005; Park et al., 2009). Our data are consistent with the proposed role of aPKC as a key component of the apical complex, as we found ACD correlates with assembly of the PAR3/aPKC apical complex. However, recent analyses of ACD in the epidermis of mice lacking PKC λ do not support an essential role for aPKC in ACD (Niessen et al., 2013). Determining whether these phenotypic differences are due to compensation by other aPKC in mice lacking PKC λ or due to the role of PDK1 in heterologous signaling pathways will be important for elucidating the true role of aPKC in ACD.

Finally, we demonstrate that PDK1 also is required for differentiation of keratinocytes. We found that PDK1 regulates Notch-dependent transcription programs that promote keratinocyte differentiation. In vitro, defective expression of differentiation markers in PDK1^{CKO} keratinocytes induced with calcium were rescued by the introduction of Notch ICD, demonstrating an unexpected role for PDK1 in the regulation of Notch-dependent transcriptional programs. These results show that PDK1 has multiple functions in epidermal stratification by regulating ACD

and keratinocyte differentiation. As a result, PDK1^{CKO} mice have an epidermis that exhibits a remarkable lack of stratification. Curiously, despite normal basal cellularity, we were unable to detect changes in PDK^{CKO} keratinocyte proliferation or apoptosis either in vitro or in vivo. Nevertheless, we suspect that, although we could not detect significant changes, there may be a subtle increase in apoptosis of PDK1^{CKO} cells that is masked by either rapid clearance or sloughing of dying cells, as has been observed at other epithelial surfaces (Bondow et al., 2012). In summary, PDK1, triggered via PI3K-PIP₃ signaling, is indispensable for asymmetric signaling events that promote ACD and the development of stratified epithelia.

EXPERIMENTAL PROCEDURES

Animals

PDK1^{Flox/Flox} mice (Hashimoto et al., 2006) were bred with K14-Cre transgenic mice (Dassule et al., 2000) to generate K14-Cre^{tg/+} PDK1^{Flox/+} and K14-Cre^{tg/+} PDK1^{Flox/Flox} mice. All mice were kept in specific pathogen-free conditions in the animal care facility at Columbia University. All mouse experiments were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC). Immunostaining was visualized using a Carl Zeiss Axio Imager 2 and BioRevo BZ-9000 (KEYENCE).

qPCR

For qRT-PCR, total RNA was isolated using Trizol (Invitrogen) or RNeasy Mini (QIAGEN) and reverse transcribed by Superscript III (Invitrogen) or miRCURY LNA Universal RT miRNA PCR cDNA synthesis kit (Exiqon). cDNAs were used for PCR with SYBR Green reagents (Quanta Biosciences) on MX3000 bioanalyzer (Stratagene) and CFX Connect real-time PCR detection system (Bio-Rad). The data were normalized to β -actin expression or 5S rRNA expression for miRNA-203 expression. Product sizes were confirmed by gel electrophoresis. Primer sets for miRNA-203 and 5S rRNA were purchased from Exiqon. Additional primer sequences are provided in Table S1.

Cell Culture

Primary keratinocytes were isolated from newborn mice, cultured in low-calcium medium (0.05 mM Ca²⁺), and induced to differentiate by raising calcium to 1.3 mM, as described previously (Lichti et al., 2008). For cell-signaling experiments, cells were serum starved for 24 hr prior to stimulation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.04.051>.

AUTHOR CONTRIBUTIONS

T.D. designed all experiments, performed most of the experiments, and wrote the paper. M.S.H. analyzed data, performed some experiments, and helped in writing the paper. S.-G.P. generated the KO mice. H.O., J.J.S., Y.G.-B., and K.M.B. performed some experiments. Y.M., K.K., and T.H. analyzed data. S.G. conceived and guided the study and wrote the paper.

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